The influence of active transport systems on morphine -6-glucuronide transport in MDCKII and MDCK-PGP cells

[1]Sattari M., [2]Routledge PA., [*3]Mashayekhi SO.

[1]Tropical and Infectious Research Centre, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, [2]Department of Pharmacology, Therapeutics and Toxicology, College of Medicine, Cardiff University, Cardiff, UK, [3]Health Services Management Research Center, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

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ABSTRACT

Background and the purpose of the study: Morphine-6-glucuronide (M6G) is a potent metabolite of morphine which has high penetration into the brain despite its high polarity, which could be the result of an active transport system involved in M6G transport through blood brain barrier. Examples of such transporters are p-glycoprotein (PGP), probenecid-sensitive transport mechanism, multidrug resistance related protein 1-3, the organic anion transporter family, and the organic anion transporter polypeptide family. The aim of present study was to elucidate the mechanisms involved in transporting morphine's potent metabolite, M6G.

Methods: M6G permeability via two cell lines; MDCKII and MDCK-PGP, was compared with that of sucrose. M6G transport was examined in different concentrations and in the presence of inhibitors of different transport systems such as cyclosporine, digoxin and probenecid. M6G concentration was measured using ELISA assay. The method was sensitive, reliable and reproducible.

Results: The results confirmed that M6G could cross a layer of MDCK II or MDR-PGP cells more than sucrose could. It was also observed that M6G is a PGP transporter substrate. Its permeability was increased by the use of a PGP expressed cell line, and also in the presence of a strong PGP inhibitor. Digoxin related transporters such as Oatp2 may also involved in transport of M6G. M6G seemed to be a glucose transporter 1 substrate, but was not a substrate to probenecid sensitive transporters.

Major conclusion: It is concluded that different transporters are responsible for M6G transports via different membrane, which could have effects on its pharmacokinetics or pharmacodynamics.

Keywords: morphine-6-glucuronide, p-glycoprotein (PGP), transport.

INTRODUCTION

There are specialised transport systems mediating the influx and/or efflux of many substances. P-glycoprotein (PGP), probably is one of the best studied transporters to date. It is an ATP-binding cassette superfamily of membrane transport proteins (1), mainly present in epithelial cells in the body, where it localizes to the apical membrane. and has a great diversity in the structure of transport substrates and inhibitors (2). Besides PGP, there are other transporters (3) such as multidrug resistance related protein 1-3 (Mrp 1-3), the organic anion transporter family (Oat 1-3), and the organic anion transporter polypeptide family (Oatp 1-3).

Morphine is an important drug in the treatment of moderate to severe pain and M6G is its potent metabolite (4). Morphine passage into the blood brain barrier (BBB) is well recognised but compared to many drugs and other opioids, its penetration rate is rather limited (5). M6G high penetration into the brain despite its «high polarity» has been shown previously (6). Studies comparing morphine and M6G permeability via BBB has shown different results (7, 8). The reason for these unexpected reports could indicate that active influx and/or efflux mechanisms are involved in entrance and exit of morphine and M6G from the brain. These could suggest that M6G could be PGP substrates and if so PGP might play an important role in M6G toxicity by increasing uptake at the level of the BBB and also by decreasing renal elimination. Alternatively, the presence of PGP inhibitors might alter M6G pharmacokinetics and/or pharmacodynamics.

PGP might not be the only mechanism for transporting M6G through various membrane and there are suggestions that a probenecid-sensitive

Correspondence: mashayekhis@tbzmed.ac.ir

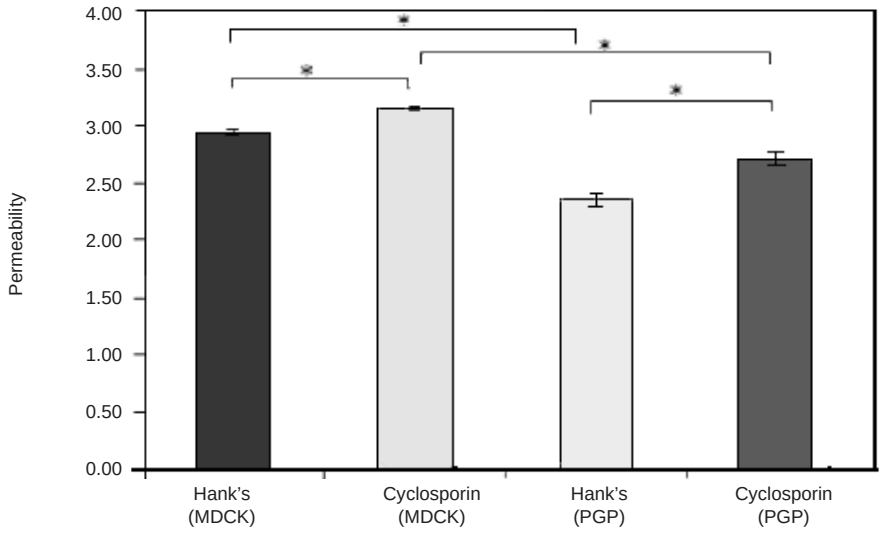

Figure 1. M6G permeability (X 10^{-6} cm/sec) in the presence of different working buffers (with/without Cyclosporin) and two different cell lines (MDCK II and MDR-PGP).
* $P < 0.001$

transport mechanism could be involved in M6G transport (7). There are also other transporters with possible effects on M6G transport of which role in M6G transport is unknown.

This study was designed to evaluate the role of PGP and other transporters in the transport of M6G across the cell monolayer using transwells containing a monolayer of MDCK II or MDR-PGP cells.

MATERIAL AND METHODS

Material and cell lines

Morphine, M6G, probenecid and cyclosporine (all from Sigma-Aldrich Co, UK). digoxin (Sigma, UK), D-glucose (BDH Chemicals Ltd. England), [6, 6'(n)- ^{3}H] sucrose (Amersham Bioscience AB), Dulbecco›s Modified Eagle's medium with high D-glucose (DMEM, Gibco-BRL), penicillin and streptomycin (Invitrogen, UK), Trypsine (Invitrogen, UK), Foetal Bovine Serum (FBS, Invitrogen, UK), and organic liquid: optiphase 'Hisafe'3 (Wallas, Perkin Elmer. Loughborough, Leics**,** England) were used in this study. Hanks' balanced salt solution and phosphate buffer solution (PBS) were made in the lab using analytical grade chemicals. Polycarbonate membrane Transwell® (6.5mm diameter, 0.4 μm Pore size, tissue culture treated, polystyrene plates, 12/plate, 48/case, sterile, Costar®, Corning Incorporated, USA).

Culture media consisted of 10% FBS, 2% penicillin/ streptomycin and 88% DMEM mixed in a sterile plastic tube and kept at 2-8°C for maximum of 7 days. Madin-Darby Canine Kidney epithelial cell lines (MDCK-II, passages 3 - 7), and MDCK I -MDR **(**MDCK-PGP or MDCK WT, passages 6 - 10). Both cells were purchased from Borst, Amsterdam.

M6G was dissolved in a solution of 10% ethanol/water (v/v) as stock solution. This stock solution was diluted to the required concentration for each experiment with working buffer, whilst ethanol concentration was kept constant (10%).

M6G assays

M6G concentration in samples was measured by ELISA assay (9). The method of measurement of M6G is very sensitive and detects small changes in M6G concentrations.

Cell preparation

Cells were cultured, spilited, and seeded upon transwells inserts similar to a previous study (10).

Transport Study

After completion of the incubation time, Transepithelial Electrical Resistance (TEER) of each transwell inserts was measured using an EVOM epithelial voltammeter (WIP, Sarasota, FL) and the transwell inserts were distributed evenly between treatment groups on the basis of TEER measurement. Then the media from inserts (both apical and basal chambers) were replaced with the working buffer for an equilibration period of 30 min prior to the start of the transport study. The transport study in the direction of A→ B was initiated by replacing 0.25 ml of apical solution with the same volume of M6G solution in the working buffer to the apical chambers (t = 0 min). The plates were stirred on an orbital shaker at a rate of 125 rpm at 73° C. A sample (0.2 ml) was removed at each interval from the basal chamber and replaced with the same amount of the working buffer (Hank's balanced salt solution) over a period of 100 min (after 15, 30, 45, 60, 80 and 100 min). Samples were also removed from the apical as well as the basal chamber at the final sampling. The

Table 1. The permeability of morphine, M6G and sucrose via MDCK II cells and MDR-PGP cells.

Permeability (X 10^{-6} cm/sec)	**MDCK II cells**	**MDR-PGP cells**	**The differences between two cell lines**
M6G (100 µg/ml)	2.94 ± 0.03, N=4	2.35 ±	P < 0.001
Sucrose (8,000,000 DPM/ml)	0.46 ± 0.09, N=4	0.38 ± 0.04, N=4	R^2 = 0.2553, P > 0.01

corrected cumulative concentrations of drug were determined based on the amount of removed and added buffer in each series of wells at each interval and the corrected concentrations were used for calculation of the apparent permeability coefficient (ρ) according to the equation (10):

$$dM/dt = \rho \mathbf{X} A \mathbf{X} C_0,$$

where dM/dt is the cumulative mass (M) of drug transported from donor to receiver chambers over the time course of the experiment, *p* is the permeability coefficient (centimeters per second), A represents the surface area of transwell membrane and C_0 represents the initial concentration of substance in the donor chamber assumed to remain essentially constant (i.e., < 5% loss) throughout the experiment.

Effect of concentration of M6G transport using MDCK II cells

Three concentrations of M6G (1, 10, 100 µ g / ml) was prepared in Hank's balanced salt solution and content of apical chambers were replaced by 0.25 ml of these solutions (time = 0 min). M6G concentrations of all samples and morphine concentrations were also measured in both apical and basal chambers of final sampling (time = 100 min) were measured in order to show or reject any possible de-glucuronidation of M6G into morphine. Transport of same concentrations of M6G via MDCK II cells using DMEM as working buffer was compared with results of Hank's balanced salt solution experiment.

Sucrose transport via MDCK II and MDR-PGP cells

Sucrose permeability was compared to that of M6G via MDCK II and MDR-PGP cells (n=4). Radiolabeled sucrose (8,000,000 dpm/ml) was dissolved in DMEM and this solution replaced the buffer in apical chamber. Sampling was performed by removing 0.2 ml from the basal and replacing the same volume with DMEM at 15, 30, 45, 60, 80 and 100 min time intervals.

Identification M6G as PGP substrate

Two methods were used to show that M6G is a PGP substrate; one was comparing M6G transport via MDCK II and MDR-PGP cells (n = 4) and the second was to study the effects of cyclosporine (10 µM), a potent PGP inhibitor (10) on M6G transport.

Morphine transport via MDCK II cells in the presence of other drugs

The effects of probenecid (10 µM), d-glucose (5 µ) and morphine (100 µg/ml) on M6G transport was examined separately. Each treatment group comprised of n=4 replicates. These results were compared with those of an experiment without above drugs and with M6G (6.182 µg/ml) solutions in Hank's buffer.

Statistical Analyses

Prism software (ver. 2) was used for the analysis of the data. One way ANOVA test was used to examine the differences between permeability of M6G under different conditions. A significant level of P < 0.05 was adopted for all tests.

RESULTS

M6G crossing MDCK II cells and MDR-PGP cells

M6G was shown to be able to cross a layer of MDCK II cells and MDR-PGP cells more than sucrose could. M6G permeability was 6.5 times higher than sucrose permeability via MDCK II cells (P < 0.01). The permeability was higher for both compounds via MDCK II cells compared with MDR-PGP cells but in the case of sucrose, the difference failed to reach to a significant level (Table 1). Using a sensitive morphine assay (11) it was shown that there was not a significant or detectable morphine in apical and basal chambers at t= 100 min.

Effect of the M6G concentration

M6G permeability stayed unchanged significantly by changes in its concentration (R^2 = 0.102, P = 0.793). M6G transports was significantly different when DMEM and Hank's buffer were used as working buffer (R^2 = 0.984, P = 0.0001, Table 2).

Identification as PGP substrate

A comparison of permeability via MDCK II and MDR-PGP cells showed that M6G permeability was significantly lower in MDR-PGP cells compared to MDCK II cells (2.35 ± 0.06 X 10^{-6} cm/sec *vs.* 2.94 ± 0.03 X 10^{-6} cm/sec, P < 0.001).

Cyclosporine inhibits PGP activity and reduces its efflux activity and therefore increases the transport of PGP substrates (12). Cyclosporine increased

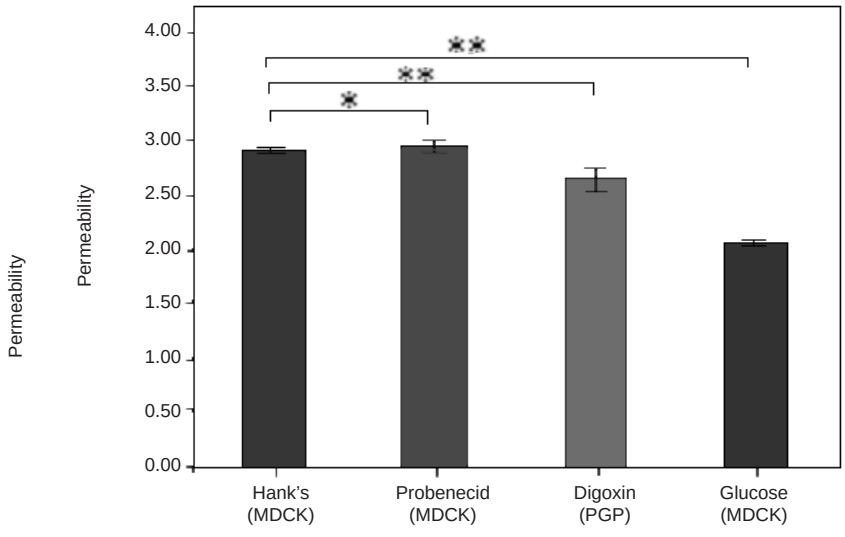

Figure 2. Effects of the presence of various transporters substances on M6G permeability (X 10^{-6} cm/sec) via MDCKII cell lines (*P > 0.05 and ** P < 0.05).

Table 2. The permeability of M6G using Hank's buffer and DMEM as working buffer in the presence of MDCK II.

M6G Concentration (μg/ml)	Permeability ± SD	
	Hank's buffer	DMEM
1.0	2.99 ± 0.34	2.01 ± 0.06
10.0	3.06 ± 0.16	2.15 ± 0.06
100.0	2.92 ± 0.02	2.11 ± 0.08

Table 3. The permeability of M6G in the presence of morphine in the presence of MDCK II.

	Permeability ± SD (x10^{-6}. cm/Sec)
M6G(100 μg/ml)	2.92 ± 0.02
M6G (100 μg/ml) + morphine (61,820 ng/ml)	3.31 ± 0.14

M6G permeability in MDCK II cells significantly from 2.94 ± 0.03 X 10^{-6} cm/sec to 3.16 ± 0.01 X 10^{-6} cm/sec (P < 0.001, Fig 1). When cyclosporine was added to MDR-PGP cells, M6G permeability also increased significantly (2.71 ± 0.04 X 10^{-6} cm/sec, P < 0.001).

M6G transport via MDCK II cells in the presence of other drugs

Probenecid (10μ M) had no significant effect on M6G permeability (2.95 ± 0.06 X 10^{-6} cm/sec, P > 0.05). Addition of d-glucose (5μ M) and digoxin (5μ M) significantly reduced M6G permeability via MDCK II cells (2.06±0.02 X 10^{-6} cm/sec, 2.64± 0.11 X 10^{-6}.cm/Sec, respectively, P < 0.05). Figure 2 show the effects of these compounds on M6G transport. Morphine (61,820 ng/ml) also did not have a significant effect on M6G transport (R^2 = 0.019, P = 0.287, Table 3).

DISCUSSION

The amount of apical to basal transepithelial movement of M6G was 6.5 times higher than sucrose and 10 times lower than that of morphine (10). This difference was much smaller than it could be explained by higher lipophilicity of morphine (4) and it seems that there may be an efflux system that lower M6G permeability. Unlike morphine (10), M6G permeability was not affected significantly by its concentration.

MDR-PGP cells were significantly less permeable to M6G than MDCK II cells which proved that M6G is a PGP substrate. Furthermore it was shown that M6G permeability increased significantly in the presence of cyclosporine; which is a potent PGP inhibitor. which is in agreement with previous finding. The effect of cyclosporin on permeability was greater in the presence of MDR-PGP cells compared to MDCK II cells.

It has been shown that probenecid enhances CNS uptake of M3G by increasing its influx into rats brain (13) and similar results was expected because of the structural similarity of M6G to M3G. However in the present study the same results that has been reported for M6G brain uptake in presence of probenecid was not obtained (3).

A threefold reduction in M6G brain uptake in mice in the presence of digoxin has been reported previously (14) which was in agreement with the results of the current study. If digoxin only worked as a PGP inhibitor, there should be an increase in M6G permeability but digoxin is transported by PGP and Oatp2. Oatp2 transporters are placed at both the luminal and basolateral sides of the brain epithelial cell and act on influx and efflux (14). This transporter could explain the observed results.

It has been reported that unlike most peptides, glycopeptide enkephalins analogues could cross the BBB, reach receptors and produce analgesia in mice (15). The suggested transporter is the transporters of d-glucose, which are located in the endothelial cell membranes which compose the BBB (15). In this study, addition of d-glucose caused a significant reduction in M6G permeability which could suggest that M6G is also a substrate for GLUT-1. The structural similarity of the glucuronic part of the M6G molecule to glucose is a possible explanation and since morphine does not possess this group, it could not be affected by d-glucose presence (10). The difference in M6G permeability in the presence of the two different working buffers which were different in glucose contents is a further proof of previous finding. It has been shown that morphine did not have an effect on M6G permeability which means that morphine is not a PGP inhibitor.

Previously it was suggested that morphine and M6G pharmacokinetic and pharmacodynamics could be influenced by age (16) and/or presence of other medications with effects on their protein bindings (17, 18). This study shows another possible factor that could affect M6G brain uptake, pharmacokinetic and pharmacodynamics.

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